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ANALYSIS OF CORTISOL, METHYLPREDNISOLONE, AND METHYLPREDNISOLONE HEMISUCCINATE

ABSENCE OF EFFECTS OF TROLEANDOMYCIN ON ESTER HYDROLYSIS

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SUMMARY

A sensitive, selective, and reproducible high-performance liquid chromatographic assay for the simultaneous measurement of cortisol and methylprednisolone using dexamethasone as the internal standard is presented. Samples are extracted with methylene chloride, washed with sodium hydroxide and then water, and chromatographed on a microparticle silica gel column with ultraviolet detection at 254 nm. Sensitivity is greater than 10 ng/ml and the intra-day coefficient of variation is less than 5% for both steroids. The use of porcine liver esterase allows the quantitation of the hemisuccinate ester of methylprednisolone. This assay has been applied in pharmacokinetic studies including investigations of troleandomycin—methylprednisolone interactions. A typical plasma concentration—time profile for methylprednisolone and its ester prodrug is presented for one subject before and after receiving troleandomycin therapy. Although methylprednisolone elimination is reduced in the presence of troleandomycin therapy, there is no effect on the pharmacokinetics of methylprednisolone sodium succinate.

INTRODUCTION

Methylprednisolone is an important glucocorticoid for the treatment of various diseases. This steroid has particular utility in therapy of acute bronchospasm (status asthmaticus) when it is administered as its water-soluble sodium succinate ester [1—4]. Inter-patient variability in the therapeutic response to methylprednisolone therapy and the occurrence of unexplained adverse effects warrant the examination of methylprednisolone disposition in selected

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patients. Thus, assays for methylprednisolone are needed for use in pharmacokinetic studies including bioavailability assessment and the examination of altered steroid disposition in the presence of disease or as a result of drug—steroid interactions [4]. An assay which can simultaneously measure endogenous cortisol in addition to serum methylprednisolone may further aid in the characterization of the therapeutic response to and side effects from methylprednisolone therapy. The measurement of serum cortisol has the added advantage of monitoring the recovery of the adrenal-pituitary axis during withdrawal of steroid therapy.

Because the active steroids are of primary interest, the analysis of the ester prodrug should not diminish the sensitivity, specificity or reliability of the methylprednisolone and cortisol assay. Analytic techniques for the simultaneous measurement of methylprednisolone and its ester prodrug have been reported [5, 6]; however, they are either non-specific or do not have the ability to simultaneously analyze cortisol and methylprednisolone. Other normal-phase high-performance liquid chromatographic (HPLC) assays for methylprednisolone and cortisol have been presented [7, 8]. In addition to assaying the hemisuccinate ester, this assay has an improved prechromatography extraction procedure, has obviated some potential problems with the internal standards, and has allowed further in vitro and in vivo studies of the drug interaction between methylprednisolone and troleandomycin [4].

EXPERIMENTAL

Materials

The HPLC system consisted of a continuous flow, constant volume Model 6000A solvent delivery system and a Model 440 UV absorbance detector (Waters Assoc., Milford, MA, U.S.A.). Injections were made onto the system with a Model 7125 universal loop injector (Rheodyne, Berkeley, CA, U.S.A.). The UV absorbance of all steroids was measured at 254 nm. A Zorbax SIL (Dupont, Wilmington, DE, U.S.A.) column (25 cm × 4.6 mm I.D., 5—6 μm particle size) equipped with a 70 × 6 mm stainless-steel Whatman precolumn was used to separate the compounds. The precolumn consisted of HC-Pellocil (37—53 μm particles) (Whatman, Clifton, NJ, U.S.A.). The methylene chloride and hexane used in the extraction procedure and mobile phase were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The pure ethanol (U.S.P.) employed in the mobile phase was obtained from U.S. International Chemicals (New York, NY, U.S.A.). The glacial acetic acid, also used in the mobile phase, was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.).

The methylprednisolone and methylprednisone were gifts of UpJohn (Kalamazoo, MI, U.S.A.). Methylprednisolone hemisuccinate was purchased from Steraloids (Wilton, NH, U.S.A.). Cortisol, dexamethasone, and carboxylic ester hydrolase (EC 3.1.1.1) Type II were obtained from Sigma (St. Louis, MO, U.S.A.). Pharmaceutical grade decolorizing carbon, neutral, was purchased from Amend Drug and Chemical (Irving, NJ, U.S.A.). Anhydrous sodium sulfate, 1 *M* sodium hydroxide solution, sodium phosphate monobasic and sodium phosphate dibasic were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Standard preparation

Decolorizing carbon (12.5 g) was added to 240 ml of pooled human plasma and the mixture stirred for 2 h at room temperature. The carbon was then removed by centrifugation for 6 h at 17,000 *g* at 4°C. Finally, the plasma was filtered through a 0.45-μm Millipore filter to remove carbon fines. To this cortisol-stripped plasma, standards of cortisol and methylprednisolone in acetonitrile—methanol (1:1) were added to provide concentrations of 25—500 ng/ml.

Extraction procedure

Plasma samples (up to 1 ml) were added to acid-washed glass extraction tubes with PTFE-lined screw caps (20 × 150 mm). If plasma samples were less than 1 ml, sufficient distilled water was added to bring the total sample volume to 1 ml. The internal standard, dexamethasone (250 ng in 80 μl of methanol) was mixed with the sample, 15 ml of methylene chloride were added, and the tubes were shaken for 20 min. The tubes were centrifuged and the aqueous layer and creamy interface aspirated. The organic phase was then washed with 1 ml of 0.1 *M* sodium hydroxide and subsequently with 1 ml of distilled water. After aspirating the aqueous phase, 1 g of anhydrous sodium sulfate was added to dry the methylene chloride. The latter was evaporated to dryness at 45°C under a nitrogen gas stream.

Chromatography

The residue was reconstituted with approximately 200 μl of mobile phase for injection. The mobile phase consisted of a hexane—methylene chloride—ethanol—acetic acid (26:69:3.4:1) mixture and the flow-rate was maintained at 2 ml/min.

Steroid recovery

The assay recovery of each steroid was assessed at 50 and 500 ng/ml in the following manner. Ten plasma samples (1 ml) containing each steroid were extracted and injected onto the chromatograph. Ten injections of the same amount of steroid (e.g. 50 and 500 ng) in mobile phase were directly injected. The peak heights of the steroids in both sets of samples were measured. The assay recovery of each steroid was computed using the following equation:

$$\text{Percent recovery} = \frac{\text{Peak height, extracted drug}}{\text{Mean peak height, direct injection}} \times 100$$

Methylprednisolone hemisuccinate hydrolysis

A hydrolysis study was performed to assess the time required for complete hydrolysis of the hemisuccinate to methylprednisolone. Human plasma (8 ml) was spiked with methylprednisolone hemisuccinate at a concentration of 1000 ng/ml, 72 units of carboxylic-ester hydrolase were then added to the plasma and 1-ml samples were obtained over a 50-min period. The hydrolysis reaction was terminated by immediately extracting the samples with methylene chloride.

Quantitation of methylprednisolone hemisuccinate

Prior to the addition of the internal standard in the extraction procedure, 80 μl containing 9 units of carboxylic-ester hydrolase in 0.5 *M* sodium phosphate buffer, pH 7.4 were added to the samples. The samples were incubated with the enzyme for 30 min at 37°C. After incubation, the internal standard was added and the samples were carried through the extraction procedure. The concentration of methylprednisolone hemisuccinate was calculated as the difference between methylprednisolone concentrations from hydrolyzed and unhydrolyzed samples.

REPRODUCIBILITY

The within-day and between-day reproducibility of the assay was assessed for methylprednisolone and cortisol at 50 and 500 ng/ml concentrations. The intra-day coefficient of variation for measurement of methylprednisolone hemisuccinate was also determined at these concentrations in the presence of 500 ng/ml methylprednisolone.

Patient studies

The following procedure was utilized to evaluate the effect of troleandomycin therapy on methylprednisolone sodium succinate hydrolysis. Corticosteroid pharmacokinetics were studied in an 11-year-old female asthmatic patient before and one week after initiating a course of troleandomycin therapy. The study dose consisted of 40 mg methylprednisolone sodium succinate (Solu-Medrol, Upjohn) administered over 2 min via an intravenous catheter. The study conditions were identical except for the oral administration of troleandomycin 250-mg capsules (TAO, Roerig) every 6 h for one week before and on the day of the second corticosteroid study. Blood samples were obtained from another intravenous catheter prior to and at selected times following the intravenous corticosteroid dose. This catheter was placed on the arm contralateral to that of the injection site. Blood samples were immediately centrifuged and plasma stored at −20°C until analysis. Since the patient was receiving steroid therapy for the treatment of her disease, endogenous cortisol secretion was suppressed resulting in plasma cortisol concentrations below the detection limits of our assay. To illustrate the ability of this assay to simultaneously measure methylprednisolone and cortisol, corticosteroid pharmacokinetics were examined after the administration of 20 mg of methylprednisolone sodium succinate (Solu-Medrol, Upjohn) to a 31-year-old normal male volunteer at 9 a.m. Steroid administration and sample collection were the same as the above patient study.

RESULTS

A chromatogram resulting from the HPLC analysis of natural human plasma is presented in Fig. 1a. Endogenous cortisol elutes as the last peak. Fig. 1b illustrates the response to steroid concentrations of approximately 250 ng/ml in charcoal-stripped human plasma from which endogeneous steroids were removed. Each steroid eluted with sharp peaks and distinct separation at base-

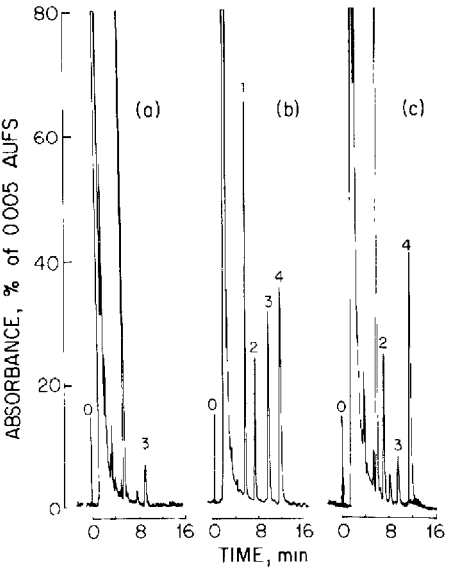

Fig. 1. Chromatograms of (a) blank plasma extract; (b) cortisol-stripped plasma spiked with 250 ng of steroids; (c) plasma extract obtained 0.5 h after a single 20-mg intravenous dose of methylprednisolone hemisuccinate. Peaks: (1) methylprednisone; (2) internal standard, dexamethasone: (3) cortisol; (4) methylprednisolone. The symbol 0 designates the injection point.

line. A chromatogram resulting from the HPLC analysis of plasma obtained 0.5 h after the intravenous injection of 20 mg of methylprednisolone hemisuccinate to a human subject is shown in Fig. 1c. This chromatogram represents concentrations of 103 ng/ml for cortisol and 398 ng/ml for methylprednisolone. Each steroid of interest is defined clearly. An endogenous compound which elutes between dexamethasone and cortisol is prominent in natural plasma, but does not interfere.

The steroid extraction recoveries at two concentrations and sensitivity limits of the assay are presented in Table I. The mean assay recoveries of methylprednisolone, cortisol, and dexamethasone were about 62% and were independent of concentration. The recovery of each of these steroids is also independent of the amount of plasma contained in the initial aqueous phase. Thus, diluted plasma samples may be assayed along with undiluted samples utilizing the same standard curve. Such dilution of plasma is desirable to bring the peak height ratio within the calibration plot range for samples of high concentrations. This dilution is also necessary if the initial aqueous:organic phase ratio is to be maintained when limited volumes of plasma are available. The equivalent extraction recovery of dexamethasone compared with cortisol and methylprednisolone substantiates the suitability of this steroid as an internal standard in this assay procedure.

The apparent minimum quantitation limit for both methylprednisolone and cortisol is 10 ng/ml. The minimum detection limit for these steroids is appproximately 2 ng/ml. The latter concentration cannot be quantified, but reflects the lower limit of steroid concentration producing a detector response.

Calibration curves of peak height ratio versus steroid concentration are linear

TABLE I

STEROID EXTRACTION RECOVERIES AND ASSAY SENSITIVITY LIMITS

Steroid	Percent recovery			Minimum limits (ng/ml)	
	50 ng/ml	500 ng/ml	Mean	Quantitation**	Detection
Methylprednisolone	62.6 (4.5)*	61.3 (5.7)	62.0	10	2
Cortisol	62.8 (4 5)	59.8 (5.3)	61.2	10	2
Dexamethasone	64.2 (4.7)	60.4 (5.5)	62.3	—	—

*Mean (S.D.).
**Based on a signal-to-noise ratio of 2.5.

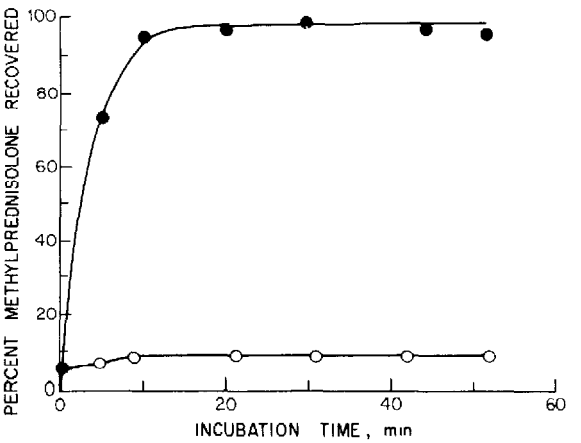

Fig. 2. Time course of hydrolysis of methylprednisolone hemisuccinate in the presence (•) and absence (○) of carboxylic ester hydrolase

over the range 10—1000 ng/ml for both cortisol and methylprednisolone. However, cortisol produces a slightly greater detector response than does methylprednisolone.

The hydrolysis rate of methylprednisolone hemisuccinate is shown in Fig. 2. Methylprednisolone hemisuccinate is hydrolysed rapidly and completely to its free alcohol within the incubation time for this assay. The lack of progressive hydrolysis of the ester in the absence of carboxylic ester hydrolase demonstrates the stability of the ester in plasma. Similar results are observed if the hydrolysis is performed in whole blood in the absence of the esterase. Neither troleandomycin nor oleandomycin in plasma samples interfere with the rate and extent of hydrolysis catalyzed by this enzyme.

The intra-day and inter-day variability of the assay for methylprednisolone and cortisol are presented for high and low steroid concentrations in Table II. Both coefficients of variation are less than 5% for the two steroids. Since the hemisuccinate ester is quantified by difference, intra-day variability of the assay for this ester was determined in the presence of 500 ng/ml methylprednisolone. Under these conditions, the variability in determination of the steroid ester is comparable with the free alcohol. The relatively high variability

TABLE II

INTRA-DAY AND INTER-DAY COEFFICIENTS OF VARIATION

All variability statistics are based on ten measurements.

Steroid	Intra-day coefficient of variation (%)		Inter-day coefficient of variation (%)	
	50 ng/ml	500 ng/ml	50 ng/ml	500 ng/ml
Methylprednisolone	1.6	3 7	3.5	1.6
Cortisol	2.2	1.9	4.7	4.2
Methylprednisolone hemisuccinate*	17.3	3.6	—	—

*In the presence of methylprednisolone, 500 ng/ml.

TABLE III

RELATIVE RETENTION TIMES OF SELECTED GLUCOCORTICOIDS

Retention times relative to methylprednisolone, corrected for column hold-up time.

Steroid	Relative retention time (min)
Fluocinonide	0.29
Cortisone	0.34
Corticosterone	0.35
Methylprednisone	0.37
Prednisone	0.44
Beclomethasone	0.51
Dexamethasone	0.54
Betamethasone	0.63
Cortisol	0 77
Methylprednisolone	1.00
Prednisolone	1.03

at the 50 ng/ml concentration is expected since the difference between the hydrolysed and unhydrolysed concentration is less than 10%.

The assay was examined for selectivity by injecting other endogenous and synthetic glucocorticoids onto the chromatographic system. The relative retention times of these materials are listed in Table III. The assay method clearly separates this series of closely related compounds. Many other conjugated and oxidized metabolites of these steroids probably do not warrant concern as the initial organic extraction step excludes the lipid-insoluble biotransformation products from the HPLC column. This assay has been utilized for the determination of methylprednisolone concentrations in patients receiving other therapeutic agents [4]. These drugs include: theophylline, terbutaline, isoproterenol, epinephrine, phenobarbital, phenytoin, troleandomycin, erythromycin, and hydroxyzine. None of these drugs or their metabolites interfered with this assay.

The conversion of methylprednisolone hemisuccinate to its free alcohol is rapid with maximal concentrations of the latter found at 30 min (Fig. 3A

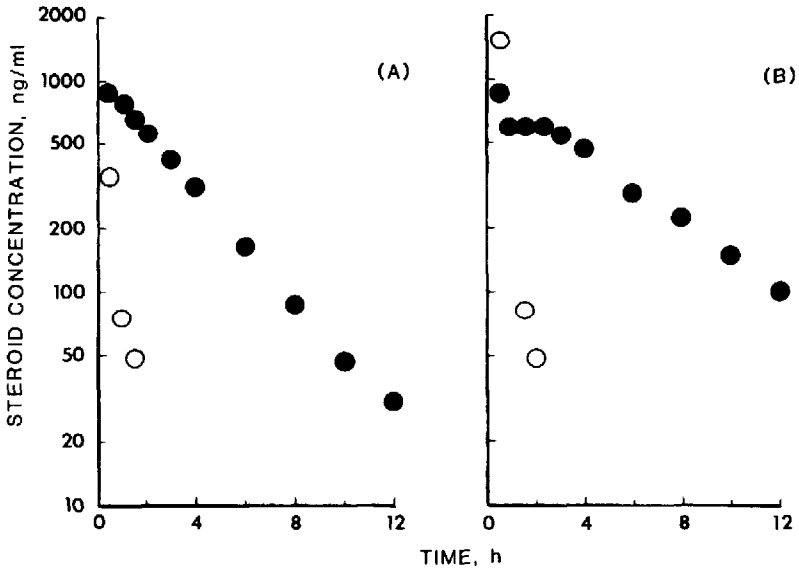

Fig. 3. Plasma methylprednisolone (•) and methylprednisolone hemisuccinate (○) concentrations after a single intravenous dose of 40 mg of methylprednisolone hemisuccinate given to an 11-year-old asthma patient before (A) and after (B) administration of 250 mg of TAO every 6 h for one week.

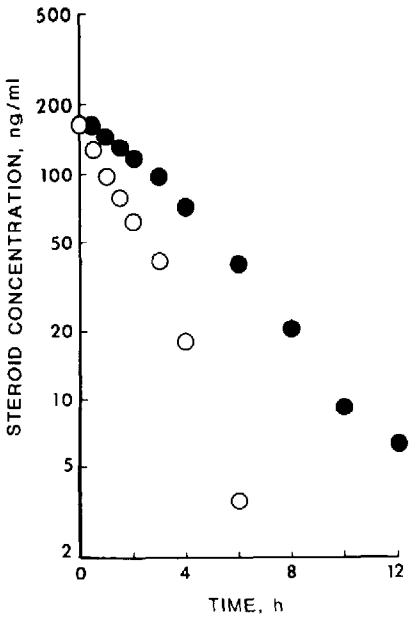

Fig. 4. Plasma methylprednisolone (•) and endogenous cortisol (○) concentrations after a single intravenous dose of 20 mg of methylprednisolone hemisuccinate given to a 31-year-old normal male volunteer at 9 a.m.

and B). When TAO is co-administered, the ester behaves similarly, although inhibition of methylprednisolone disposition was found. In the baseline study methylprednisolone is eliminated with a half-life of 2.3 h and plasma clearance

of 358 ml/min per 1.73 m^2. While this patient was receiving TAO, the elimination half-life and plasma clearance were 3.3 h and 248 ml/min/1.73 m^2.

The simultaneous measurement of cortisol and methylprednisolone is illustrated in Fig. 4. The zero h (9 a.m.) cortisol concentration was 180 ng and rapidly fell below assay detection limit by 4 p.m. (7 h). Although the 9-a.m. value is within normal limits (60 to 260 ng/ml) the 4-p.m. value in normal subjects is typically between 20 and 180 ng/ml. This marked decline in cortisol concentrations is indicative of suppression of cortisol secretion by methylprednisolone.

DISCUSSION

The simultaneous measurement of methylprednisolone and cortisol by this HPLC method is efficient, precise, sensitive and selective. To date, over 2000 samples have been analyzed by this method in studies of the disposition kinetics of methylprednisolone and cortisol. These compounds are stable in frozen serum for extended periods. Some serum samples have been repeatedly assayed at time intervals of twelve months or more and yield identical steroid concentrations.

Incubation of samples with carboxylesterase allows determination of the hemisuccinate ester of methylprednisolone by the difference of results with and without use of the enzyme. A lower quantitation limit for the analysis of the ester has not been presented since this limit is determined primarily by the relative difference in methylprednisolone and total methylprednisolone concentrations. With this method, methylprednisolone hemisuccinate concentrations are considered non-quantifiable if the relative difference is less than 10%.

The hemisuccinate ester does not partition into methylene chloride because the extraction of plasma is carried out under neutral pH conditions. This affords subsequent treatment of the methylene chloride extract with acidic or basic washes without having the assay confounded by hydrolysis of the ester to the active steroid. Although we have not found any interference from co-administered drugs or their metabolites, the ability to rigorously wash the extract provides a method of circumventing interference if the problem arises.

Extractability of the internal standard from aqueous solutions is identical with the steroids of interest. Other published HPLC assays for methylprednisolone do not share this feature. One reversed-phase assay has been reported which utilizes an endogenous glucocorticoid, cortexolone, as an internal standard [5]. However, cortexolone extraction recovery was lower than that of methylprednisolone. This assay method also does not allow simultaneous measurement of methylprednisolone and cortisol. A normal-phase assay has been reported which employs the acetate ester of methylprednisolone as the internal standard [7]. Although the extractability of this ester was not provided, the water—octanol and water—diethyl ether partition coefficients of this ester are probably ten-fold greater than methylprednisolone or cortisol [9]. Additionally, because this internal standard is an ester, the extraction process and subsequent treatment of the extract may require mild conditions to prevent its hydrolysis to methylprednisolone. Another assay for methylprednisolone utilizes an internal standard which is added after extraction [8].

Much of the utility of the internal standard in correcting for recovery and other sources of variation is lost at that stage of the procedure.

Pharmacokinetic studies of methylprednisolone have been carried out using a radioimmunoassay technique [6, 10]. Cross-reactivity with the major metabolite of this steroid, methylprednisone, was reported and may complicate the results as patients attain appreciable plasma concentrations of methylprednisone [4]. The radioimmunoassay method also does not permit concomitant assay for cortisol. While the present assay is unable to resolve methylprednisone, it has a shorter retention time and does not interfere with measurement of cortisol or methylprednisolone.

The diminished apparent clearance and prolonged half-life of methylprednisolone during co-administration of troleandomycin have been reported [4]. This drug combination is often beneficial in treatment of patients with severe steroid-dependent asthma [1—3]. However, mechanisms responsible for this drug interaction have not been fully elucidated. Since methylprednisolone must be cleaved from its ester prodrug before it appears in plasma, alterations in ester disposition were of concern. TAO is also converted to active forms through de-esterification and competition with hydrolysis of methylprednisolone hemisuccinate require consideration. The determination of plasma concentrations of methylprednisolone hemisuccinate along with the active steroid (Fig. 3) indicates that the in vivo disposition of the ester appears to be unaltered in the presence of TAO. The in vitro hydrolysis of the ester was similarly unaffected by the presence of the antibiotic. Thus, the inhibition of methylprednisolone elimination is unlikely to be due to altered hydrolysis of the ester.

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